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(54) Title: ENHANCING THE CIRCULATING HALF-LIFE OF ANTIBODY-BASED FUSION PROTEINS

(57) Abstract: Disclosed are compositions and methods for enhancing the circulating half-life of antibody-based fusion proteins. Disclosed methods and compositions rely on altering the amino acid sequence of the junction region between the antibody moiety and the fused protein moiety in an antibody-based fusion protein. An antibody-based fusion protein with an altered amino acid sequence in the junction region has a greater circulating half-life when administered to a mammal. Disclosed methods and compositions are particularly useful for reducing tumor size and metastasis in a mammal.

ENHANCING THE CIRCULATING HALF-LIFE OF ANTIBODY-BASED FUSION PROTEINS

Related Applications

This application claims priority to, and the benefit of U.S.S.N. 60/181,768, filed February 11, 2000, the disclosure of which is incorporated by reference herein.

Field of the Invention

The present invention relates generally to fusion proteins. More specifically, the present invention relates to methods of enhancing the circulating half-life of antibody-based fusion proteins.

Background of the Invention

The use of antibodies for treating human diseases is well established and has become more sophisticated with the introduction of genetic engineering. Several techniques have been developed to improve the utility of antibodies. These include: (1) the generation of monoclonal antibodies by cell fusion to create "hybridomas", or by molecular cloning of antibody heavy (H) and light (L) chains from antibody-producing cells; (2) the conjugation of other molecules to antibodies to deliver them to preferred sites *in vivo*, e.g., radioisotopes, toxic drugs, protein toxins, and cytokines; (3) the manipulation of antibody effector functions to enhance or diminish biological activity; (4) the joining of other proteins such as toxins and cytokines with antibodies at the genetic level to produce antibody-based fusion proteins; and (5) the joining of one or more sets of antibody combining regions at the genetic level to produce bi-specific antibodies.

Proteins can be joined together through either chemical or genetic manipulation using methods known in the art. See, for example, Gillies *et al.*, Proc. Natl. Acad. Sci. USA 89:1428-1432 (1992); and U.S. Patent No. 5,650,150.

However, the utility of recombinantly-produced antibody-based fusion proteins may be limited by their rapid *in vivo* clearance from the circulation. Antibody-cytokine fusion proteins, for example, have been shown to have a significantly lower *in vivo* circulating half-life than the free antibody. When testing a variety of antibody-cytokine fusion proteins, Gillies *et al.* reported that all of the fusion proteins tested had an α phase (distribution phase) half-life of less than 1.5 hours. Indeed, most of the antibody-based fusion proteins were cleared to 10% of the serum concentration of the free antibody by two hours. See, Gillies *et al.*, BIOCONJ. CHEM. 4: 230-235

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(1993). More recently, it was shown that antibody-based fusion proteins with reduced binding affinity for an Fc receptor have enhanced circulating half-lives. It was also shown that a reduced binding affinity for the Fc receptor interfered with some of the antibody effector functions such as antibody-dependent cellular cytotoxicity (ADCC), but did not interfere with other functions such as complement fixation or antigen binding. See Gillies *et al.*, Cancer Res. 59(9):2159-66 (1999).

In some cases, such as the treatment of cancer or viral diseases, it would be desirable to maintain antibody effector functions and long circulating half-life. Therefore, there is a need in the art for additional methods of enhancing the *in vivo* circulating half-life of antibody-based fusion proteins.

Summary of the Invention

Immunoglobulin G (IgG) molecules interact with multiple classes of cellular receptors including three classes of Fc γ receptors (Fc γ R) specific for the IgG class of antibody, namely Fc γ RI, Fc γ RII and Fc γ RIII. They also interact with the FcRp class of receptor in a pH-dependent manner with little or no binding at neutral pH but high binding at a pH of 6.0.

The serum half-life of an antibody is influenced by the ability of that antibody to bind to an Fc receptor (FcR) and to the Fc protection receptor (FcRp). The serum half-life of immunoglobulin fusion proteins is also influenced, for example, by the ability to bind to such receptors (Gillies *et al.*, Cancer Res. 59:2159-66 (1999)).

The invention discloses the surprising observation that, within fusion proteins comprising an immunoglobulin (Ig) moiety and a non-immunoglobulin (non-Ig) moiety, alteration of amino acids near the junction of the two moieties dramatically increases the serum half-life of the fusion protein. The observation is surprising because the amino acid changes affect protein surfaces that are distinct from the interaction surfaces of the Fc region with the Fc receptors and with the Fc protection receptor. In addition, the amino acid changes of the invention have their effect even when the known Fc receptor and Fc protection receptor are not primarily determining the serum half-life of the fusion protein. Thus, the amino acid alterations of the invention can be combined with amino acid alterations affecting the interaction with Fc receptor and/or Fc protection receptor to achieve synergistic effects.

The present invention provides fusion proteins containing an immunoglobulin in which the serum half-life is improved as a result of alterations that are at sites distinct from the Fc region's interaction surface with Fc receptor and Fc protection receptor (FcRp). The present

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invention also provides methods for the production of fusion proteins between an immunoglobulin moiety and a second, non-immunoglobulin protein having an improved serum half-life.

The alterations in the amino acid sequence of the fusion protein are preferentially at the junction of the Ig moiety and the non-Ig moiety. The junction region of the fusion protein contains alterations that, relative to the naturally occurring sequences of the Ig heavy chain and non-Ig protein, preferably lie within about 10 amino acids of the junction point. More preferably, the amino acid changes cause an increase in hydrophobicity. Even more preferably, the amino acid changes involve changing the C-terminal lysine of the antibody moiety to a hydrophobic amino acid such as alanine or leucine. In a preferred embodiment, the fusion protein of the invention comprises an Ig heavy chain, preferably located N-terminal to a second, non-Ig protein.

In another embodiment of the invention, the binding affinity of fusion proteins for FcRp is optimized by alteration of the interaction surface of the Fc moiety that contacts FcRp. The important sequences for the binding of IgG to the FcRp receptor have been reported to be located in the CH2 and CH3 domains. According to the invention, alterations of the fusion junction in a fusion protein are combined with alterations of Fc's interaction surface with FcRp to produce a synergistic effect. In some cases it may be useful to increase the interaction of the Fc moiety with FcRp at pH 6, and it may also be useful to decrease the interaction of the Fc moiety with FcRp at pH 8. Such modifications include alterations of residues necessary for contacting Fc receptors or altering others that affect the contacts between other heavy chain residues and the FcRp receptor through induced conformational changes. Thus, in a preferred embodiment, an antibody-based fusion protein with enhanced *in vivo* circulating half-life is obtained by first linking the coding sequences of an Ig constant region and a second, non-immunoglobulin protein and then introducing a mutation (such as a point mutation, a deletion, an insertion, or a genetic rearrangement) in an IgG constant region at or near one or more amino acid selected from Ile 253, His 310 and His 435. The resulting antibody-based fusion proteins have a longer *in vivo* circulating half-life than the unmodified fusion proteins.

In certain circumstances it is useful to mutate certain effector functions of the Fc moiety. For example, complement fixation may be eliminated. Alternatively or in addition, in another set of embodiments the Ig component of the fusion protein has at least a portion of the constant region of an IgG that has reduced binding affinity for at least one of FcγRI, FcγRII or FcγRIII. For example, the gamma4 chain of IgG may be used instead of gamma1. The alteration has the

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advantage that the gamma4 chain results in a longer serum half-life, functioning synergistically with one or more mutations at the fusion junction. Accordingly, IgG2 may also be used instead of IgG1. In an alternative embodiment of the invention, a fusion protein includes a mutant IgG1 constant region, for example an IgG1 constant region having one or more mutations or deletions of Leu₂₃₄, Leu₂₃₅, Gly₂₃₆, Gly₂₃₇, Asn₂₉₇, or Pro₃₃₁. In a further embodiment of the invention, a fusion protein includes a mutant IgG3 constant region, for example an IgG3 constant region having one or more mutations or deletions of Leu₂₈₁, Leu₂₈₂, Gly₂₈₃, Gly₂₈₄, Asn₃₄₄, or Pro₃₇₈. However, for some applications, it may be useful to retain the effector function that accompanies Fc receptor binding, such as ADCC.

10 In a preferred embodiment, the second, non-immunoglobulin moiety of the fusion protein is a cytokine. The term "cytokine" is used herein to describe naturally occurring or recombinant proteins, analogs thereof, and fragments thereof which elicit a specific biological response in a cell which has a receptor for that cytokine. Preferably, cytokines are proteins that may be produced and excreted by a cell. Cytokines preferably include interleukins such as interleukin-2 (IL-2), IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-14, IL-15, IL-16 and IL-18, hematopoietic factors such as granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF) and erythropoietin, tumor necrosis factors (TNF) such as TNF α , lymphokines such as lymphotoxin, regulators of metabolic processes such as leptin, interferons such as interferon α , interferon β , and interferon γ , and chemokines. Preferably, the antibody-cytokine fusion protein of the present invention displays cytokine biological activity.

20 In an alternative preferred embodiment, the second, non-immunoglobulin moiety of the fusion protein is a ligand-binding protein with biological activity. Such ligand-binding proteins may, for example, (1) block receptor-ligand interactions at the cell surface; or (2) neutralize the biological activity of a molecule (*e.g.*, a cytokine) in the fluid phase of the blood, thereby preventing it from reaching its cellular target. Preferably, ligand-binding proteins include CD4, CTLA-4, TNF receptors, or interleukin receptors such as the IL-1 and IL-4 receptors. Preferably, the antibody-receptor fusion protein of the present invention displays the biological activity of the ligand-binding protein.

30 In yet another alternative preferred embodiment, the second, non-immunoglobulin moiety of the fusion protein is a protein toxin. Preferably, the antibody-toxin fusion protein of the present invention displays the toxic activity of the protein toxin.

In yet other preferred embodiments, the second, non-immunoglobulin moiety of the fusion protein is a hormone, neurotrophin, body-weight regulator, serum protein, clotting factor,

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protease, extracellular matrix component, angiogenic factor, anti-angiogenic factor, or another secreted protein or secreted domain. For example, CD26, IgE receptor, polymeric IgA receptor, other antibody receptors, Factor VIII, Factor IX, Factor X, TrkA, PSA, PSMA, Flt-3 Ligand, endostatin, angiostatin, and domains of these proteins.

5 In yet other embodiments, the second, non-immunoglobulin moiety is a non-human or non-mammalian protein. For example, HIV gp120, HIV Tat, surface proteins of other viruses such as adenovirus, and RSV, other HIV components, parasitic surface proteins such as malarial antigens, and bacterial surface proteins are preferred. These non-human proteins may be used, for example, as antigens, or because they have useful activities. For example, the second, non-
10 immunoglobulin moiety may be streptokinase, staphylokinase, urokinase, tissue plasminogen activator, or other proteins with useful enzymatic activities.

According to the invention, the non-immunoglobulin moiety can be a portion of a protein. Preferably, the non-Ig protein moiety is a protein portion that substantially retains the functional and or structural properties of an intact protein. In a preferred embodiment, the non-
15 Ig protein moiety is a functional or structural portion of a protein described herein.

In a preferred embodiment, the antibody-based fusion protein comprises a variable region specific for a target antigen as well as a constant region, either of which is linked through a peptide bond to a second, non-immunoglobulin protein. The constant region may be the constant region normally associated with the variable region, or a different one, *e.g.*, variable and constant
20 regions from different species. The heavy chain may include any combination of one or more CH1, CH2, or CH3 domains. Preferably, the heavy chain includes CH1, CH2, and CH3 domains, and more preferably, only CH2 and CH3 domains. In one embodiment, the antibody-based one fusion protein comprises an Fv region with fused heavy and light chain variable regions. Also embraced within the term "fusion protein" are constructs having a binding domain
25 comprising framework regions and variable regions (*i.e.*, complementarity determining regions) from different species, such as are disclosed by Winter, *et al.*, Great Britain Patent No. 2,188, 638. Antibody-based fusion proteins comprising a variable region preferably display antigen-binding specificity. In yet another preferred embodiment, the antibody-based fusion protein further comprises a light chain. The invention thus provides fusion proteins in which the
30 antigen-binding specificity and activity of an antibody are combined with the potent biological activity of a second, non-immunoglobulin protein, such as a cytokine. A fusion protein of the present invention can be used to deliver selectively the second, non-immunoglobulin protein to a target cell *in vivo* so that the second, non-immunoglobulin protein can exert a localized biological effect.

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In an alternative preferred embodiment, the antibody-based fusion protein comprises a heavy chain constant region linked through a peptide bond to a second, non-immunoglobulin protein, but does not comprise a heavy chain variable region. The invention thus further provides fusion proteins which retain the potent biological activity of a second, non-immunoglobulin protein, but which lack the antigen-binding specificity and activity of an antibody.

In preferred embodiments, the fusion protein comprises two chimeric chains comprising at least a portion of a heavy chain and a second, non-Ig protein linked by a disulfide bond.

In preferred embodiments, the fusion proteins of the invention are useful to treat cancer, viral infections, immune disorders, and to enhance the growth (including proliferation) of specific cell types.

The invention also features DNA constructs encoding the above-described fusion proteins, and cell lines, *e.g.*, myelomas, transfected with these constructs.

These and other objects, along with advantages and features of the invention disclosed herein, will be made more apparent from the description, drawings, and claims that follow.

Brief Description of the Drawings

Figure 1 shows the pharmacokinetic behavior of the KS-IL-2 fusion protein and various mutant fusion proteins containing substitutions of the antibody heavy chain's C-terminal lysine moiety or other alterations described in the Examples. Levels of antibody or fusion protein were measured by an ELISA that tests for IL-2 (Figure 1A) or human Fc (Figure 1B).

Figure 2 shows the pharmacokinetic properties of KS-IL-2 fusion proteins carrying either the gamma1 or gamma4 chain with either the wild-type lysine or the lysine-to-alanine mutation at the C-terminus of the antibody heavy chain. Levels of antibody or fusion protein were measured by an ELISA that tests for the IL-2 moiety.

Figure 3 shows the pharmacokinetic properties of fusions of a human antibody to Tumor Necrosis Factor alpha (TNFalpha). Levels of fusion protein were measured by an ELISA that tests for the human Fc region. Shown are the levels of an intact antibody-TNFalpha fusion protein (black diamonds) and the levels of an otherwise identical fusion protein in which the C-terminal lysine of the antibody moiety has been deleted (gray squares).

Figure 4 shows the binding of antibody-IL-2 fusion proteins to membranes of fixed J774 cells, which are rich in the FcγR class of receptor. Shown are the binding of a non-mutant KS-

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IL-12 fusion protein (black diamonds) and a KS-IL-12 fusion protein carrying a mutation of the heavy chain C-terminal Lysine to Alanine (gray squares).

Figure 5 shows the effect of antibody-cytokine fusion protein treatment of Balb/C mice bearing subcutaneous tumors derived from CT26 colon carcinoma cells that were engineered to express human EpCAM, the antigen for KS.

Detailed Description of the Invention

The present invention provides antibody fusion proteins having one or more mutations at the junction between the Ig and non-Ig moieties which increase the circulating half lives of the fusion proteins. The mutant fusion proteins of the invention have the advantageous property that their serum half-life is improved without affecting the interaction of the antibody moiety with either of the two known pharmacokinetic-determining receptors in the body: Fc receptor and FcRp.

In general, an antibody-based fusion protein of the invention comprises a portion of an immunoglobulin (Ig) protein joined to a non-immunoglobulin (non-Ig) protein, such that the amino acid sequence of the region spanning the junction between the Ig and non-Ig proteins has at least one mutation when compared to the wild-type amino acid sequences of the Ig and non-Ig proteins.

In one embodiment, at least one mutation is in the C-terminal region of the Ig portion. In another embodiment, at least one mutation is in the N-terminal region of the non-Ig protein. In a further embodiment, the fusion protein contains at least one mutation in the C-terminal region of the Ig portion, and at least one mutation in the N-terminal region of the non-Ig protein. A mutation may be a point mutation, an insertion, a deletion, or a gene rearrangement. In preferred embodiments the mutation increases the hydrophobicity of the junction region. For example, the mutation replaces a charged or ionizable amino acid with a non-charged or hydrophobic amino acid (e.g., a Lys, Arg or other ionizable residue is replaced with an Ala, Leu, Gly, Trp or other non-charged or hydrophobic residue).

In an optional embodiment, a spacer or linker peptide is inserted between the Ig and non-Ig proteins. The spacer or linker peptide is preferably non-charged, more preferably non-polar, and or hydrophobic. The length of a spacer or linker peptide is preferably between 1 and about 100 amino acids, more preferably between 1 and about 50 amino acids, or between 1 and about 25 amino acids, and even more preferably between 1 and about 15 amino acids. In another embodiment of the invention, the Ig and non-Ig moieties of the fusion protein are joined via a

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spacer or linker peptide, and there is at least one mutation in either one or both of the Ig and non-Ig moieties. In an alternative embodiment of the invention, the Ig and non-Ig moieties are separated by a synthetic spacer, for example a PNA spacer, that is preferably non-charged, more preferably non-polar, and or hydrophobic.

5 According to the invention, an immunoglobulin (Ig) chain is an immunoglobulin protein or a portion of an immunoglobulin protein that includes a variable or a constant domain. An Ig chain is preferably a portion of an immunoglobulin heavy chain, for example, an immunoglobulin variable region capable of binding a preselected cell-type. In a preferred embodiment, the Ig chain comprises a variable region specific for a target antigen as well as a
10 constant region. The constant region may be the constant region normally associated with the variable region, or a different one, *e.g.*, variable and constant regions from different species. In a more preferred embodiment, an Ig chain includes a heavy chain. The heavy chain may include any combination of one or more CH1, CH2, or CH3 domains. Preferably, the heavy chain includes CH1, CH2, and CH3 domains, and more preferably only CH2 and CH3 domains. In
15 one embodiment, the portion of the immunoglobulin includes an Fv region with fused heavy and light chain variable regions.

 According to the invention, a non-immunoglobulin protein includes a naturally occurring protein that is not an immunoglobulin, or a synthetic or recombinant protein that is not an immunoglobulin, or a fragment of any of the above. In a preferred embodiment, a non-
20 immunoglobulin protein includes a functional domain such as a ligand binding domain, an enzymatic domain, a regulatory domain, or a domain that interacts with one or more cellular factors. In an alternative embodiment, a non-immunoglobulin domain comprises a structural domain or an epitope.

 In a preferred embodiment, the Ig chain is joined to the non-Ig protein via a gene fusion.
25 Preferably, the gene fusion is synthetic or recombinant, and is generated using standard techniques of chemical synthesis or molecular biology. Typically, a mutation is introduced as part of the gene fusion construct. Alternatively, a mutation may be introduced subsequently, using known methods of mutagenesis (for example by exposing the gene fusion construct to irradiation, or chemical or biological mutagenesis).

30 According to the invention, a junction region is the region of the fusion protein surrounding the junction point between the Ig and non-Ig moieties of the fusion protein. In a preferred embodiment, the junction region includes the C-terminal portion of the Ig moiety and the N-terminal portion of the non-Ig moiety. In one embodiment, the junction region also

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comprises a spacer or linker peptide inserted at the junction point between the Ig and non-Ig moieties.

According to preferred embodiments of the invention, a mutation in the Ig moiety is in the C-terminal portion of the Ig moiety, preferably within about 100 residues, more preferably within about 50 residues, or about 25 residues, and even more preferably within about 10 residues from the C-terminus of the Ig moiety.

According to preferred embodiments of the invention, a mutation in the non-Ig moiety is in the N-terminal portion of the non-Ig moiety, preferably within about 100 residues, more preferably within about 50 residues, or about 25 residues, and even more preferably within about 10 residues from the N-terminus of the non-Ig moiety.

In preferred embodiments of the invention, a mutation is in the C-terminal region of the Ig moiety, but the mutation is not in part of the Ig protein that interacts with the Fc receptor (FcR) or FcRp.

An antibody fusion protein having a mutation according to the invention has an increased *in vivo* circulating half-life when compared to the circulating half-life of a corresponding antibody fusion protein without the mutation. The circulating half-life of an antibody fusion protein can be measured by assaying the serum level of the fusion protein as a function of time.

Experimental evidence indicates that the effects of preferred mutations of the invention are not dependent on interactions with FcR or FcRp. First, preferred mutations that increase the circulating half-life of a fusion protein do not affect regions of the antibody that, on the three dimensional structure, are part of the interaction surface that binds to FcR or to FcRp. Second, preferred mutations of the invention can cause an improvement in serum half-life even when the interaction with FcR is removed by use of an IgG-gamma4 chain and the interaction with FcRp is removed by performing the pharmacokinetic study in a beta2-microglobulin mutant mouse in which FcRp is defective. Third, preferred mutations of the invention do not significantly affect the binding of Ig fusion proteins to FcR on J774 cells.

Site-directed mutagenesis analyses indicate that the surface of Fc that interacts with the Fc receptor is near the hinge region on the CH2 domain. The Fc region's FcR interaction surface is very far, in three dimensions, from the C-terminus of Fc. Similar analyses indicate that FcRp interacts with amino acid residues located at the interface between the CH2 and CH3 domains.

FcRp binds its ligand with a much higher affinity at acidic pH (pH 6.0), than at neutral or slightly basic pH (pH 7.4). This is consistent with the role of FcRp in protecting Fc containing molecules such as antibodies following their cellular internalization within endosomes. These cellular compartments become acidified after fusion with lysosomes and their protein

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constituents are degraded by acidic proteases. Binding to membrane bound FcRp during this process prevents degradation of the antibody and allows it to be recycled to the outside of the cell (back into the circulation) or across a cell layer (a process called transcytosis). This latter process allows IgG to pass through the neonatal intestinal mucosa following the ingestion of milk in the acidic environment of the gut.

The structure of the Fc/FcRp complex indicates that FcRp binds to the side of the Fc region, with contacts in both the CH2 and CH3 domains, and that the contacted region is not particularly close to the C-terminus of the Fc region. Thus, alteration of the very C-terminal region of the Fc is not expected to alter the interaction with FcRp.

Not wishing to be bound by any particular theory, it is believed that mutations in the fusion junction region that increase the circulatory half life of a fusion protein according to the invention also reduce cleavage of the fusion protein in a protease cleavage assay, as illustrated in Example 15. It is further believed that protease digestion may contribute to the disappearance of intact proteins from the body, including fusion proteins. Thus, resistance to proteases may directly contribute to improved pharmacokinetics of proteins. It is also further believed that protease digestion of non-denatured proteins involves access by a protease to an exposed sequence in the correct conformation, as well as recognition of a specific sequence of amino acids. Thus, mutations in the fusion junction that affect the general conformation of a protein and thus affect accessibility of proteases to their cleavage sites may contribute to protease resistance and to improved pharmacokinetics. In addition, mutations that alter specific protease recognition sequences may contribute to protease resistance and to improved pharmacokinetics.

A feature of mutations of the invention is that they can be combined with other mutations or substitutions in the antibody moiety to synergistically modulate serum half-life or other properties of the Ig moiety. For example, one or more mutations of the invention that increase the circulating half-life of an antibody fusion protein can be combined with one or more mutations that affect the interaction between the antibody fusion protein and FcR or FcRp.

In addition, the mutations of the invention can be used with a wide variety of antibody moieties and with a wide variety of non-Ig fusion partners. The immunoglobulins include IgG, IgM, IgA, IgD, and IgE. The non-Ig fusion partners include cytokines, other secreted proteins, enzymes, or soluble fragments of transmembrane receptors, such as ligand-binding domains.

According to the invention, an antibody-based fusion protein with an enhanced *in vivo* circulating half-life can be further enhanced by modifying within the Fc portion itself. These may be residues including or adjacent to Ile 253, His 310 or His 435 or other residues that can effect the ionic environments of these residues when the protein is folded in its 3-dimensional

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structure. The resulting proteins can be tested for optimal binding at pH 6 and at pH 7.4-8 and those with high levels of binding at pH 6 and low binding at pH 8 are selected for use *in vivo*. Such mutations can be usefully combined with the junction mutations of the invention.

5 Methods and compositions of the invention are useful when coadministered with angiogenesis inhibitors such as those disclosed in PCT/US99/08335 (WO 99/52562) or prostaglandin inhibitors such as those disclosed in PCT/US99/08376 (WO 99/53958). Methods and compositions of the invention can also be used in multiple cytokine protein complexes such as those disclosed in PCT/US00/21715. Methods and compositions of the invention are also useful in combination with other mutations disclosed in PCT/US99/03966 (WO 99/43713) that
10 increase the circulating half-life of a fusion protein.

Non-limiting methods for synthesizing useful embodiments of the invention are described in the Examples herein, as well as assays useful for testing pharmacokinetic activities, both *in vitro* and in pre-clinical *in vivo* animal models. The preferred gene construct encoding a chimeric chain includes, in 5' to 3' orientation, a DNA segment which encodes at least a portion
15 of an immunoglobulin and DNA which encodes a second, non-immunoglobulin protein. An alternative preferred gene construct includes, in 5' to 3' orientation, a DNA segment which encodes a second, non-immunoglobulin protein and DNA which encodes at least a portion of an immunoglobulin. The fused gene is assembled in or inserted into an expression vector for transfection of the appropriate recipient cells where it is expressed.

20 The invention also provides methods for identifying mutations that increase the circulatory half-life of an antibody-based fusion protein. The methods comprise introducing one or more mutations in a region spanning the junction between the Ig moiety and the non-Ig moiety of an antibody-based fusion protein. The circulating half-life of the mutated fusion protein is assayed, preferably by monitoring its serum level *in vivo* as a function of time.

25 In one embodiment of the invention, a mutation that increases the circulatory half-life of an antibody-based fusion protein is a mutation that reduces cleavage of the fusion protein in a protease cleavage assay, as discussed in Example 15. The mutation is preferably a mutation in a region spanning the junction between the Ig moiety and the non-Ig moiety of the fusion protein (for example, a mutation in the junction region discussed above). Alternatively, the mutation
30 may be any mutation in the fusion protein that reduces protease cleavage and increases the circulatory half life of the fusion protein, as described in Example 16. Accordingly, the invention provides methods for screening mutations in proteins in general, and preferably in an Ig-cytokine fusion protein, to identify mutations that increase the circulatory half-life of the fusion protein.

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The invention is illustrated further by the following non-limiting examples. The amino acid residue numbers used herein refer to the IgG1 amino acid sequence. One of ordinary skill in the art will understand that corresponding mutations in fusion proteins involving other Ig proteins are useful to increase their circulating half-lives.

5 Accordingly, the teachings presented herein are applicable to other Ig molecules such as IgG2, IgG3, IgG4, IgA, IgM, IgD, or IgE.

Examples

Example 1. Construction of antibody-IL-2 genes with substitutions of the Lys codon at the fusion junction

10 The amino acid sequence at the junction of the antibody-IL-2 fusion protein is SerProGlyLys-AlaProThr (SEQ ID NO: 1), in which the SerProGlyLys (SEQ ID No. 2) is the normal carboxy terminus of the heavy chain of the antibody, and AlaProThr is the N-terminal sequence of mature IL-2. In order to determine the effect alterations in the region of the fusion junction on the pharmacokinetics of the fusion protein, substitutions or deletion of the residue
15 were made by mutagenesis, as described below.

The expression vector for immunocytokines was described in Gillies *et al.*, (1998) J. Immunol. 160:6195-6203. In the human gamma-1 gene encoding the heavy chain, the XmaI restriction site located 280 bp upstream of the translation stop codon was destroyed by introducing a silent mutation (TCC to TCA). Another silent mutation (TCT to TCC) was
20 introduced to the Ser codon three residues upstream of the C-terminal lysine of the heavy chain to create the sequence TCC CCG GGT AAA (SEQ ID No. 3), which contains a new XmaI site [Lo *et al.*, (1998) Protein Engineering 11:495-500]. The IL-2 cDNA was constructed by chemical synthesis and it contains a new and unique PvuII restriction site [Gillies *et al.*, (1992) Proc. Natl. Acad. Sci. 89:1428-1432]. Both the XmaI and PvuII sites are unique in the
25 expression vector, and they facilitated mutagenesis of the lysine codon which lies at the junction of the CH3 and the IL-2 DNA.

Substitution or deletion of the Lys codon was achieved by replacing the XmaI-PvuII fragment in the immunocytokine expression vector with an oligonucleotide duplex encoding the desired mutation. In this case the variable regions of the heavy and light chains were derived
30 from the humanized KS antibody, which recognized a human antigen called EpCAM (Epithelial cell adhesion molecule). The sequences of the oligonucleotide duplexes used in the present invention are listed below, where the codons in bold encode the desired mutations, and the

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sequences in italics, *CCCGG* and *CAG* are the cohesive end of the *XmaI* site and the blunt end of the *PvuII* site, respectively. The oligonucleotide duplex with 5'-hydroxyl ends were used in the ligation to the *XmaI*-*PvuII* digested expression vector. The use of oligonucleotides with 5'-hydroxyl ends eliminated self ligation of the oligonucleotide duplex.

5

1.) Lys to Ala Substitution

5' *CCG GGT GCA GCA CCT ACT TCA AGT TCT ACA AAG AAA ACA CAG*
 3' (SEQ ID NO: 4)
 10 3' *CA CGT CGT GGA TGA AGT TCA AGA TGT TTC TTT TGT GTC*
 5' (SEQ ID NO: 5)

2.) Lys to Arg Substitution

15 5' *CCG GGT AGG GCG CCA ACT TCA AGT TCT ACA AAG AAA ACA CAG* 3'
 (SEQ ID NO: 6)
 3' *CA TCC CGC GGT TGA AGT TCA AGA TGT TTC TTT TGT GTC* 5'
 (SEQ ID NO: 7)

20

A *NarI* restriction site (*GGCGCC*) was also introduced by silent mutation to facilitate screening of recombinant clones.

3.) Deletion of Lys

25 5' *CCG GGT GCA CCT ACT TCA AGT TCT ACA AAG AAA ACA CAG* 3'
 (SEQ ID NO: 8)
 3' *CA CGT GGA TGA AGT TCA AGA TGT TTC TTT TGT GTC* 5'
 30 (SEQ ID NO: 9)

4.) Lys to Gly Substitution

35 5' *CCG GGT GGG GCC CCT ACT TCA AGT TCT ACA AAG AAA ACA CAG* 3'
 (SEQ ID NO: 10)
 3' *CA CCC CGG GGA TGA AGT TCA AGA TGT TTC TTT TGT GTC* 5'
 (SEQ ID NO: 11)

40 An *Apal* restriction site (*GGGCCC*) was also introduced by silent mutation to facilitate screening of recombinant clones.

5.) Lys to Leu Substitution

45 5' *CCG GGT CTG GCG CCA ACT TCA AGT TCT ACA AAG AAA ACA CAG* 3'
 (SEQ ID NO: 12)

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3' CA GAC CGC GGT TGA AGT TCA AGA TGT TTC TTT TGT GTC
 5' (SEQ ID NO: 13)

5 A NarI restriction site (GGCGCC) was also introduced by silent mutation to facilitate screening of recombinant clones.

6.) Lys to AlaAlaAla Substitution

10 5' CCG GGT GCA GCA GCT GCC CCA ACT TCA AGT TCT ACA AAG AAA ACA
 CAG 3' (SEQ ID NO: 14)
 3' CA CGT CGT CGA CGG GGT TGA AGT TCA AGA TGT TTC TTT TGT
 GTC 5' (SEQ ID NO: 15)

15

7.) Lys to Cys Substitution

5' CCG GGT TGC GCA CCA ACT TCA AGT TCT ACA AAG AAA ACA CAG 3'
 (SEQ ID NO: 16)
 20 3' CA ACG CGT GGT TGA AGT TCA AGA TGT TTC TTT TGT GTC
 5' (SEQ ID NO: 17)

25

A FspI restriction site (TGCGCA) was also introduced by silent mutation to facilitate screening of recombinant clones.

8.) Lys to Asp Substitution

30 5' CCG GGT GAC GCA CCA ACT TCA AGT TCT ACA AAG AAA ACA CAG 3'
 (SEQ ID NO: 18)
 3' CA CTG CGT GGT TGA AGT TCA AGA TGT TTC TTT TGT GTC 5'
 (SEQ ID NO: 19)

35

The recombinant gene constructs containing the various substitutions or deletion of the Lys codon were confirmed by DNA sequencing.

Example 2. Construction of antibody-IL-2 genes encoding extra amino acid residues at the fusion junction

40

It is common in the art to separate domains in fusion proteins with flexible linkers containing amino acid residues such as glycine and serine. The importance of the spacing between the CH3 and IL-2 was studied in the following mutagenesis experiments. Blunt ended oligonucleotide duplexes encoding different number of amino acid residues were inserted into the SmaI endonuclease restriction site (same recognition site as the XmaI mentioned above) of

- 15 -

the huKS-IL-2 expression vector by ligation; and the correct orientation of insertion was confirmed by DNA sequencing. As discussed above, oligonucleotide duplexes with 5'-hydroxyl ends were used to eliminate self ligation.

5

9.) Lys to Cys Substitution with linker ligation

The following linker (oligonucleotide duplex) was inserted into the SmaI endonuclease restriction site of the huKS-IL-2 expression vector by ligation. The sequence GCATGC encodes
10 a SphI restriction site, which facilitated screening of recombinants containing the linker insertion.

15

```

5'   G GCA TGC GG           3'
3'   C CGT ACG CC           5'

```

After linker ligation into the SmaI site (CCCGGG), the sequence at the fusion junction became

```

      C CCG GCA TGC GGG GGT AAA      (SEQ ID NO: 20)  (linker sequence
underlined)
20      Pro Ala Cys Gly Gly Lys      (SEQ ID NO: 21)

```

Therefore, the linker put a Cys residue at the original position of the Lys residue, for a possible interchain disulphide bond formation. The original Lys residue was pushed back by 3 amino acid residues (AlaCysGly).

25

10.) A linker encoding 6 amino acid residues

The following linker (oligonucleotide duplex) was inserted into the SmaI endonuclease restriction site of the huKS-IL-2 expression vector by ligation. The sequence GGATCC encodes
30 a BamHI restriction site, which facilitated screening of recombinants containing the linker insertion.

```

5'   G GGT TCA GGA TCC GGA GG      3'   (SEQ ID NO: 22)
3'   C CCA AGT CCT AGG CCT CC      5'   (SEQ ID NO: 23)

```

35

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After linker ligation into the SmaI site, the sequence at the fusion junction became ProGlySerGlySerGlyGlyLys (SEQ ID NO: 24), where the six amino acid residues inserted were underlined.

5 11.) a linker encoding 11 amino acid residues

The following linker (oligonucleotide duplex) was inserted into the SmaI endonuclease restriction site of the huKS-IL-2 expression vector by ligation. The sequence GGATCC encodes a BamHI restriction site, which facilitated screening of recombinants containing the linker
10 insertion.

5' G GGT TCA GGC TCT GGA TCA GGG TCC GGA TCC GG 3' (SEQ
ID NO: 25)
15 3' C CCA AGT CCG AGA CCT AGT CCC AGG CCT AGG CC 5' (SEQ
ID NO: 26)

After linker ligation into the SmaI site, the sequence at the fusion junction became ProGlySerGlySerGlySerGlySerGly SerGlyGlyLys (SEQ ID NO: 27), where the eleven amino
20 acid residues inserted were underlined.

Example 3. Construction of antibody-IL-2 genes with substitutions of the Pro codon at the fusion junction

25 The proline in the sequence ProGlyLys at the carboxyl terminus of CH3 is mutated to Ala, Leu or Gly, and other amino acids. This is accomplished by replacing a 25 base-pair SmaI-SmaI fragment of the KS-IL-2 expression vector by an oligonucleotide duplex encoding the desired change. Each of the following oligonucleotide duplexes has a SmaI cohesive end (3-base overhang) and a blunt end (for ligating to the SmaI end of the restriction fragment). The
30 substitutions at the Pro codon are denoted in bold. These substitutions had no significant effect on the pharmacokinetics of the fusion protein, indicating that the structural properties of the Pro residue have no significant effect on the pharmacokinetics of the fusion protein

12.) Pro to Ala Substitution
35 5' CG CAG AAG AGC CTC TCC CTG TCC **GC** 3' (SEQ ID NO: 28)
3' TC TTC TCG GAG AGG GAC AGG **CG** 5' (SEQ ID NO: 29)

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13.) Pro to Leu Substitution

5' CG CAG AAG AGC CTC TCC CTG TCC CT 3' (SEQ ID NO: 30)

5 3' TC TTC TCG GAG AGG GAC AGG GA 5' (SEQ ID NO: 31)

12.) Pro to Gly Substitution

5' CG CAG AAG AGC CTC TCC CTG TCC GG 3' (SEQ ID NO: 32)

10 3' TC TTC TCG GAG AGG GAC AGG CC 5' (SEQ ID NO: 33)

Example 4. Construction of hu14.18-(Lys to Ala)-IL-2 DNA

In order to show that the effect of the Lys to Ala substitution on the pharmacokinetics of the antibody-IL-2 fusion protein was not limited to the huKS antibody, we chose a different antibody, humanized 14.18 (hu14.18), which recognized GD2, a ganglioside overexpressed on the surface of many human tumor cells. The expression vector for hu14.18-(Lys to Ala)-IL-2 was constructed as described above.

20 Example 5. Construction of huKS-(deleted Lys)-TNF α DNA

In order to show that the effect of the Lys residue on the pharmacokinetics of the antibody-IL-2 fusion protein was applicable to other cytokines, we chose a different cytokine, TNF α . The complete cDNA sequence of TNF α was published by Nedwin *et al.* in *Nucleic Acids Res.* (1985) 13:6361-6373, and the expression of an antibody-TNF α also has been described by Gillies *et al.* in *Bioconjugate Chem.* (1993) 4:230-235. The fusion junction of the antibody-TNF α has the sequence SerProGlyLys-ValArgSerSerSer (SEQ ID NO: 34), where Val is the N-terminal residue of the mature TNF α . In order to compare with huKS-TNF α , DNA encoding huKS-(deleted Lys)-TNF α was constructed by an overlapping PCR method [Daugherty *et al.*, (1991) *Nucleic Acids Res.* 19:2471-2476] with mutagenic primers encoding the deletion of the Lys residue. The resultant expression vector for huKS-(deleted Lys)-TNF α therefore encodes the peptide sequence SerProGly-ValArgSerSerSer (SEQ ID NO: 35) at the fusion junction. Additional modifications of this fusion protein according to the new invention might include the removal of the Arg residue in the amino terminal sequence of TNF to further reduce the overall charge of the junction region.

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Example 6. Construction of huKS-(EU)-(Lys to Ala)-IL-2 DNA

All the antibody-cytokine fusion proteins mentioned in the examples above were based on a certain allotype of the human IgG1 represented by the myeloma H chain, KOL. In order to show that the effect of the Lys to Ala substitution on the pharmacokinetics of the antibody-IL-2 fusion protein was not limited to KOL, we chose a different IgG1 allotype represented by the myeloma H chain, EU. The EU allotype differs from the KOL allotype in three amino acid residues in the constant regions. The EU allotype contains Lys-229 at the end of CH1, and Asp-356 and Leu-358 at the beginning of CH3. The KOL allotype contains Arg-229, Glu-356 and Met-358 at the corresponding positions. The DNA encoding the EU allotype was obtained by mutagenesis of the KOL DNA using the overlapping PCR method. The resultant EU DNA was then used to replace the corresponding fragment of the KOL DNA to generate the expression vector for producing huKS-(EU)-(Lys to Ala)-IL-2.

Example 7. Transfection of cells and Expression of Proteins

For transient transfection, the plasmid was introduced into Baby Hamster Kidney (BHK) cells by lipofection using Lipofectamine Plus (Life Technologies, Gaithersburg, MD) according to supplier's protocol.

In order to obtain stably transfected clones, plasmid DNA was introduced into the mouse myeloma NS/0 cells by electroporation. NS/0 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine and penicillin/streptomycin. About 5×10^6 cells were washed once with PBS and resuspended in 0.5 ml PBS. Ten μ g of linearized plasmid DNA were then incubated with the cells in a Gene Pulser Cuvette (0.4 cm electrode gap, BioRad) on ice for 10 min. Electroporation was performed using a Gene Pulser (BioRad, Hercules, CA) with settings at 0.25 V and 500 μ F. Cells were allowed to recover for 10 min. on ice, after which they were resuspended in growth medium and then plated onto two 96 well plates. Stably transfected clones were selected by growth in the presence of 100 nM methotrexate (MTX), which was introduced two days post-transfection. The cells were fed every 3 days for two to three more times, and MTX-resistant clones appeared in 2 to 3 weeks. Supernatants from clones were assayed by anti-Fc ELISA to identify high producers. High producing clones were isolated and propagated in growth medium containing 100 nM MTX.

For routine characterization by gel electrophoresis, antibody-cytokine fusion proteins in the conditioned media were captured on Protein A Sepharose (Repligen, Cambridge, MA) and

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then eluted by boiling in the protein sample buffer with or without 2-mercaptoethanol. After electrophoresis on an SDS gel, the protein bands were visualized by Coomassie staining. The antibody heavy chain-IL-2 and the light chain had apparent MW of about 67 and 28 kD respectively, on SDS-PAGE.

- 5 For purification, the fusion proteins bound on Protein A Sepharose were eluted in a sodium phosphate buffer (100 mM NaH₂PO₄, pH 3, and 150 mM NaCl). The eluate was then immediately neutralized with 0.1N NaOH.

Example 8. ELISA Procedures

- 10 ELISAs were used to determine the concentrations of protein products in the supernatants of MTX-resistant clones and other test samples. The anti-huFc ELISA consists of a capturing step using goat anti-human IgG (against both heavy and light chains) and a detection step using the horseradish peroxidase-conjugated F(ab')₂ fragment of goat anti-human IgG, Fc fragment specific. Therefore, the anti-huFc ELISA measures human IgG, either as an antibody by itself or
15 as a cytokine fusion protein. To determine the concentration of the intact antibody-IL-2 fusion protein, an IL-2-detection ELISA was used. It consists of the same capturing step using goat anti-human IgG (against both heavy and light chains), but the detection step uses a detection antibody directed against IL-2. In some experiments, EPCAM was used instead of a capture antibody to detect KS-IL-2 fusion proteins, since the KS antibody recognizes EPCAM. In some
20 experiments, a commercial human IL-2 ELISA detection kit was used (R&D Systems). All the different ELISA procedures involving IL-2 detection antibodies gave similar results. However, as can be seen from a comparison of Figure 1A and Figure 1B, there is a progressive loss of IL-2-immunoreactive material compared to human Fc immunoreactive material in later pharmacokinetic time points. This effect is most pronounced for fusion proteins that have the
25 poorest pharmacokinetic properties.

The anti-huFc ELISA is described in detail below.

A. Coating plates.

- 30 ELISA plates were coated with AffiniPure Goat anti-Human IgG (H+L) (Jackson Immuno Research Laboratories, West Grove, PA) at 5 µg/mL in PBS, and 100 µL/well in 96-well plates (Nunc-Immuno plate Maxisorp). Coated plates were covered and incubated at 4°C overnight. Plates were then washed 4 times with 0.05% Tween (Tween 20) in PBS, and blocked

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with 1% BSA/1% goat serum in PBS, 200 μ L/well. After incubation with the blocking buffer at 37°C for 2 hrs, the plates were washed 4 times with 0.05% Tween in PBS and tapped dry on paper towels.

5 B. Incubation with test samples and secondary antibody

Test samples were diluted to the proper concentrations in sample buffer, which contains 1% BSA/1% goat serum/0.05% Tween in PBS. A standard curve was prepared with a chimeric antibody (with a human Fc), the concentration of which was known. To prepare a standard curve, serial dilutions are made in the sample buffer to give a standard curve ranging from 125
10 ng/mL to 3.9 ng/mL. The diluted samples and standards were added to the plate, 100 μ L/well and the plate was incubated at 37°C for 2 hr. After incubation, the plate was washed 8 times with 0.05% Tween in PBS. To each well was then added 100 μ L of the secondary antibody, the horseradish peroxidase-conjugated AffiniPure F(ab')₂ fragment goat anti-human IgG, Fc fragment specific (Jackson Immuno Research), diluted around 1:120,000 in the sample buffer.
15 The exact dilution of the secondary antibody has to be determined for each lot of the HRP-conjugated anti-human IgG. After incubation at 37°C for 2 hr, the plate was washed 8 times with 0.05% Tween in PBS.

C. Development

20 The substrate solution was added to the plate at 100 μ L/well. The substrate solution was prepared by dissolving 30 mg of OPD (o-phenylenediamine dihydrochloride, 1 tablet) into 15 mL of 0.025 M Citric acid/0.05 M Na₂HPO₄ buffer, pH 5, which contained 0.03% of freshly added H₂O₂. The color was allowed to develop for 30 min. at room temperature in the dark. The developing time is subject to change, depending on lot to lot variability of the coated plates, the
25 secondary antibody, etc. Watch the color development in the standard curve to determine when to stop the reaction. The reaction was stopped by adding 4N H₂SO₄, 100 μ L/well. The plate was read by a plate reader, which was set at both 490 and 650 nm and programmed to subtract the background OD at 650 nm from the OD at 490 nm.

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Example 9. Pharmacokinetic behavior of antibody-cytokine fusion proteins carrying alterations at the fusion junction.

The fusion proteins were tested for their pharmacokinetic behavior following intravenous injection into Balb/c mice. Blood was collected from mice by retro-orbital bleeding and stored at 4°C in Eppendorf micro-centrifuge tubes. In some cases, two different ELISA methods were used to measure both the amount of human antibody and the amount of second, fused non-Ig protein remaining in the blood at various time points. Alternatively, the presence of the non-Ig moiety was inferred by Western blot analysis of pharmacokinetic time points.

Using the techniques described in the preceding examples, the KS(gamma1)-IL-2 fusion mutant proteins were injected into mice, and the effect on serum half-life was determined. Some of the results are shown in Figure 1 and Figure 2. In addition, the effect of deletion of the antibody heavy chain's C-terminal lysine was examined in an IgG(gamma1)-IL-2 fusion in which the antibody had a different binding specificity. The pharmacokinetic properties of a 14.18(Lys → Ala)-IL-2 were superior to 14.18-IL-2 to an extent that was similar to the improvement of KS(Lys → Ala)-IL-2 as compared to KS-IL-2.

For antibody-IL-2 fusions, the ranking of the effect of mutations affecting the C-terminal lysine of the heavy chain on the pharmacokinetic properties was (from best to worst): Lys → Leu ~ Lys → Ala ~ Lys → Ala₃ > Lys → (deleted) > Lys → Asp ~ Lys → Gly > Lys → (no change) ~ Lys → Cys > Lys → Arg.

The pharmacokinetic properties of KS(Lys → deleted)-TNFalpha were significantly improved as compared to KS-TNFalpha (Figure 3). The pharmacokinetic profile of the KS-TNFalpha fusion protein was unusual in that, when the levels of human antibody are measured by Fc ELISA, there was a sharp drop in the level of detected protein within the first 30 minutes, followed by a slow increase in the level of human Fc-reactive material. This effect was highly reproducible.

When pharmacokinetic samples were analyzed by Western blotting, it was found that human Fc-cross-reactive material was in the form of intact antibody; the TNF moiety had been cleaved off and lost. However, similar analysis of the KS-TNFalpha fusion protein carrying a deletion of the C-terminal lysine indicated that this protein survived primarily in an intact form, with TNF still present.

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In addition, a KS-TNFalpha fusion protein was expressed in which the first eight amino acids of the mature TNFalpha sequence were deleted. The pharmacokinetic properties of the deleted KS-TNFalpha fusion protein were superior to corresponding proteins having the entire mature TNF sequence. This is likely due to removal of the charged Arg residue at the +2 position of the mature TNF which increases the hydrophobicity of the junctional region.

Changing the heavy chain constant regions of KS(Lys → Ala)-IL-2 and KS-IL-2 from KOL to EU had no effect on the pharmacokinetic properties of either protein.

Taken together, these results indicate that mutation of the junction caused a significant improvement of the pharmacokinetic properties of Ig fusion proteins. The effect was seen with diverse antibodies, and diverse non-Ig proteins fused to an Ig moiety.

Example 10. Combining mutations at the fusion junction with a change in Ig type from gamma1 to gamma4 leads to a synergistic enhancement of serum half-life that is independent of FcRp function.

The human gamma4 Fc region binds poorly to Fc receptors. As a result, fusion proteins that comprise a gamma4 Fc region generally have a superior pharmacokinetic properties as compared to fusion proteins having the gamma1 chain. To address whether junction mutations affect pharmacokinetics through an effect on an Fc receptor interaction, an FcRp interaction, or both, the pharmacokinetic properties of gamma1- and gamma4-containing fusion proteins with or without junction mutations were examined in mice that were either normal or defective in FcRp. The results of these pharmacokinetic experiments are shown in Figure 2.

Figure 2 shows the pharmacokinetic behavior of a KS(gamma1)-IL-2 fusion protein, a KS(gamma4)-IL-2 fusion protein, a KS(gamma1)(Lys-to-Ala)-IL-2 fusion protein, and a KS(gamma4)(Lys-to-Ala)-IL-2 fusion protein. Normal mice and mutant mice defective in beta2 microglobulin were examined.

These data indicated that, in a normal mouse, the pharmacokinetics of an IgG-gamma1 antibody-IL-2 fusion protein were improved by introducing a Lys-to-Ala mutation at the C-terminus of the antibody moiety. Similarly, the pharmacokinetics of an IgG-gamma4 antibody-IL-2 fusion protein were improved by introducing a Lys-to-Ala mutation at the C-terminus of the antibody moiety. These data indicate that a junction mutation can improve the pharmacokinetic

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properties of a fusion protein that already has improved pharmacokinetics as a result of reduced Fc receptor binding.

Figure 2 also shows the pharmacokinetic properties of the same proteins when injected into mutant mice lacking the beta2-microglobulin protein, which is an essential subunit of FcRp (Junghans and Anderson, Proc. Nat Acad. Sci. (1996) 93:5512-5516). Thus, these mutant mice are defective in FcRp activity. As a result, the catabolism of antibodies is about 10-fold faster in such mutant mice than in normal mice.

The data of Figure 2 indicated that the KS (gamma1) antibody, a KS (gamma1)-IL-2 fusion protein, a KS (gamma4)-IL-2 fusion protein, a KS (gamma1)(Lys-to-Ala)-IL-2 fusion protein, and a KS (gamma4)(Lys-to-Ala)-IL-2 fusion protein all were catabolized more rapidly in the beta2-microglobulin mutant mice than in wild-type mice. However, the relative order of serum half-lives is the same for these proteins in both mouse strains: the unfused antibody has the best pharmacokinetics, followed by the KS(gamma4)(Lys-to-Ala)-IL-2 fusion protein, the KS(gamma1)(Lys-to-Ala)-IL-2 fusion protein, the KS(gamma4)-IL-2 fusion protein, with the KS(gamma1)-IL-2 fusion protein having the worst pharmacokinetic properties. If a junction mutation had its effect exclusively by changing the interaction of a fusion protein with FcRp, then in the absence of FcRp function, the junction mutation should have no effect on pharmacokinetics.

Example 11. Mutation of the junction region in an intact antibody has no effect on serum half life.

A mutation in a gene encoding the heavy chain of the intact, unfused KS antibody is engineered to change the C-terminal lysine to an alanine. The wild-type and mutant forms of KS are expressed and purified by the methods described above, and the pharmacokinetic properties are compared. The pharmacokinetic behaviors of the wild-type and mutant antibodies are found to be indistinguishable.

Example 12. Binding to Fc receptor by antibody fusion proteins with or without mutations at the fusion junction

Using a standard procedure, the binding of KS-IL-2 and KS(K-A)-IL-2 to Fc receptors was examined. No effect of the mutation was found. Fusion proteins were expressed and

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purified as described above, and were tested for their ability to bind to fixed J774 cells, which express the Fc receptor. Results are shown in Figure 4.

Example 13. Treatment of colon carcinoma in a mammal with an antibody-cytokine fusion protein containing a junction mutation.

To test whether a cytokine-antibody fusion protein with a junction mutation would be advantageous in treatment of colon carcinoma in a mammal, the following experiments were performed. CT26 is a colon carcinoma cell line derived from Balb/C mice. By standard genetic engineering techniques, this cell line was engineered to express the human epithelial cell adhesion molecule (EpCAM), which is the antigen recognized by the KS antibody; these cells are termed CT26/EpCAM cells (Gillies *et al.* Journal of Immunology (1998) 160:6195-6203).

Balb/C mice were subcutaneously inoculated with 2×10^6 CT26/EpCAM cells. When tumors reached a volume of about 50-200 cubic millimeters, mice were randomized into three groups of 7 mice for further study. Beginning at day 0, tumor-bearing mice were treated with PBS, about 10 micrograms of KS-IL2 with an IgG1 heavy chain (KS-IL2gamma1), or about 10 micrograms of KS-IL2 with an IgG1 heavy chain and the Lys to Ala mutation described in the previous examples (KS-IL2gamma1 [Lys to Ala]). Mice were injected intravenously, once per day for five days. Tumor sizes were measured with calipers.

The results of one such experiment are shown in Figure 5. In this experiment, KS-IL2gamma1 caused a significant decrease in the volume of many, but not all tumors. In six of the seven KS-IL2gamma1-treated animals, tumors were still measurable on day 21. However, in the KS-IL2gamma1(Lys to Ala)-treated animals, the tumors shrank, so that by day 21, the tumors in all seven animals were unmeasurable, and by day 16, only two of seven mice had measurable tumors. In Figure 5, black diamonds indicate average tumor volumes in mice that were injected with PBS as controls on days 0, 1, 2, 3, and 4. Filled circles indicate average tumor volumes in mice treated with 10 micrograms of KS-IL2 gamma1. Intravenous injections were performed. The x-axis indicates the number of days elapsed following the first injection; the y-axis indicates the average tumor volume in cubic milliliters.

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Example 14. Inhibition of metastasis in a mammal treated with an antibody-cytokine fusion protein containing a junction mutation.

To test whether an antibody-cytokine fusion protein could inhibit metastatic growth of tumor cells, the following experiments were performed. Lewis Lung Carcinoma (LLC) is a lung carcinoma cell line derived from C57/Bl6 mice. By standard genetic engineering techniques, this cell line was engineered to express the human epithelial cell adhesion molecule (EpCAM), which is the antigen recognized by the KS antibody; these cells are termed LLC/EpCAM cells.

C57/Bl6 mice were intravenously injected with 1×10^6 LLC/EpCAM cells. After five days, mice were randomized into three groups of 6 mice and treated with either PBS, about 20 micrograms of KS-IL2, or about 20 micrograms of KS-Ala-IL2 (KS-IL2 with a Lys to Ala change at the C-terminus of the Ig moiety). Metastases were quantitated on day 24. As indicated in the table below, the PBS-treated group had large numbers of metastases into the lungs. Animals treated with KS- γ 1-IL2 had a significantly reduced number of metastases. However, animals treated with KS- γ 1-ala-IL2 had even fewer metastases than animals treated with KS- γ 1-IL2, and in one animal, no metastases at all were detected.

| <u>Treatment Group</u> | <u>Number of Metastases</u> | <u>Lung Wt. (g)</u> |
|------------------------|------------------------------------|---------------------|
| PBS | >250, >250, >250, >250, >250, >250 | 0.92 +/- 0.14 |
| KS- γ 1-IL2 | 62, 37, 18, 17, 11, 9 | 0.27 +/- 0.04 |
| KS- γ 1-ala-IL2 | 4, 4, 3, 3, 1, 0 | 0.25 +/- 0.02 |

Taken together, Examples 13 and 14 illustrate that antibody-cytokine fusion proteins can inhibit establishment of metastases as well as growth of tumor cells at the primary site. In addition, the results indicate that antibody-cytokine fusion proteins can inhibit disease resulting from a variety of different tumor types, such as colon cancer and lung cancer. Furthermore, antibody-cytokine fusion proteins with at least one amino acid change in the linker region in accordance with the invention are more effective at inhibiting metastases and tumor growth than antibody-cytokine fusion proteins with no amino acid change in the linker region.

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Example 15. Assay of antibody fusion proteins with junction mutations for resistance to proteases.

To address whether antibody-cytokine fusion proteins with junction mutations were more or less sensitive to protease digestion, purified KS-IL2 and KS-Ala-IL2 were treated with
5 various proteases for various times, and the resulting products were analyzed by SDS-PAGE.

In one experiment, 4 micrograms of KS-IL2 and KS-Ala-IL2 were treated with 0.1 mU or 0.4 mU of Cathepsin D (Enzyme Systems, Livermore, California) for about 16 hours at 37 degrees C and analyzed by SDS-PAGE. Buffer conditions were used according to the manufacturer's instructions. When KS-IL2 was treated with 0.4 mU of Cathepsin D, about 50%
10 of the KS-IL2 heavy chain was converted to various lower molecular weight forms. The dominant digestion product had a molecular weight slightly less than that of KS-IL2 heavy chain, but much larger than the KS heavy chain. This result indicates that most of the cleavage by Cathepsin D was not taking place at the heavy chain-IL2 junction.

In contrast, when KS-Ala-IL2 was incubated with 0.4 mU of Cathepsin D under the same
15 conditions, the extent of cleavage by Cathepsin D was much less, and a band with the molecular weight of the major KS-IL2 degradation product was essentially undetectable.

In a second experiment, 4 micrograms of KS-IL2 and KS-Ala-IL2 were treated with 25 mU or 50 mU of Cathepsin L (Enzyme Systems, Livermore, California) for about 16 hours at 37 degrees C and analyzed by SDS-PAGE. Buffer conditions were used according to the
20 manufacturer's instructions. When KS-IL2 was treated with 50 mU of Cathepsin L, almost all of the KS-IL2 heavy chain was converted to various lower molecular weight forms. The dominant digestion product had a molecular weight about the same as the KS heavy chain. This result indicates that much of the cleavage by Cathepsin L was taking place near or at the heavy chain-IL2 junction.

25 In contrast, when KS-Ala-IL2 was incubated with 50 mU of Cathepsin L under the same conditions, the extent of cleavage by Cathepsin L was much less, and a band with the molecular weight of the major KS-IL2 degradation product was still the major molecular weight species observed.

In a third experiment, 4 micrograms of KS-IL2 and KS-Ala-IL2 were treated with 0.04
30 mU, 0.1 mU or 0.2 mU of plasmin (Sigma, St. Louis, Minnesota) for about 16 hours at 37 degrees C and analyzed by SDS-PAGE. Buffer conditions were used according to the manufacturer's instructions. When KS-IL2 was treated with 0.04 mU of plasmin, about 3/4 of the KS-IL2 heavy chain was converted to a lower molecular weight form with an apparent molecular weight about 30 amino acids greater than that of the KS heavy chain. When KS-IL2

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was treated with 0.2 mU of plasmin, essentially all of the KS-IL2 heavy chain was converted to a lower molecular weight form with an apparent molecular weight about 30 amino acids greater than that of the KS heavy chain. These results indicate that the cleavage of KS-IL2 by plasmin was taking place close to, but not at the heavy chain-IL2 junction.

5 In contrast, when KS-Ala-IL2 was incubated with 0.04 mU of plasmin under the same conditions, the extent of cleavage by plasmin was barely detectable. When KS-Ala-IL2 was incubated with 0.2 mU of plasmin, some uncleaved product was detected. In addition when KS-Ala-IL2 was cleaved with plasmin, a species with a molecular size about 90 amino acids greater than the KS-IL2 heavy chain accumulated to a significant extent; in the KS-IL2 digestions by
10 plasmin, this +90 species was probably rapidly cleaved to the lower molecular weight +30 species, and thus failed to accumulate. Nonetheless, the Lys-to-Ala mutation caused a significant stabilization of intact KS-IL2 in the presence of plasmin. In each case, the antibody light chain was uncleaved under the conditions used.

Taken together, these results indicated that the Lys-to-Ala mutation caused a general
15 resistance to protease cleavage, even to cleavages that do not take place at the site of the mutation. Without wishing to be bound by any particular theory, the Lys-to-Ala mutation may cause the IL-2 moiety of KS to become more resistant to proteases. Proteases may play an important role in the pharmacokinetic properties of antibody fusion proteins. For example, when antibody fusion proteins are taken up by cells bearing an Fc receptor and transported into the
20 early endosome, it may be that the antibody moiety is resistant to the proteolytic conditions used, but that the fusion partner moiety is more sensitive, resulting in partial or complete digestion of the antibody fusion protein.

Example 16. Use of protease digestion to evaluate mutations in antibody fusion proteins.

25 This example provides a general method for improving the pharmacokinetic properties of a protein. A protein is tested for its pharmacokinetic properties and also its sensitivity to proteases. Variant proteins are generated and tested for greater resistance to proteolysis. Those variants with enhanced resistance to proteolysis are then tested for their pharmacokinetic properties. It is found that the proportion of proteolysis-resistant proteins with improved
30 pharmacokinetic properties is greater than for the population of variant proteins as a whole. Some variant proteins with improved pharmacokinetic properties have one or more amino acid substitutions that do not cause a profound change in the protein structure that can be inferred by inspection of the encoding sequence, such as introduction of an N-linked glycosylation site.

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Variant proteins are generated by, for example, mutagenesis of an expression construct and isolation of clones expressing individual variant proteins. Any of a variety of mutagenesis techniques is used, including site-directed mutagenesis, random mutagenesis, PCR-mutagenesis, and mutagenesis techniques that generate hybrid sequences from related genes.

5 It is useful to use intracellular proteases, such as endosomal proteases, for these assays. Without wishing to be bound by any particular theory, it is believed that the pharmacokinetics of certain proteins, particularly proteins that are not removed by renal filtration, is determined by proteolysis that occurs upon endocytosis.

10 It is also useful to use extracellular proteases, such as trypsin, chymotrypsin, plasmin, other digestive protease, other serum proteases such as clotting factors, and tissue-specific proteases. For example, tumor-specific proteases are used to test variant proteins and identify those variants that have improved pharmacokinetic properties and stability within the tumor microenvironment. In another example, proteins that are to be orally delivered are tested for their resistance to enzymes present in the gastro-intestinal tract, such as trypsin and
15 chymotrypsin. It is found that variant proteins with enhanced resistance to gastro-intestinal enzymes have improved pharmacokinetic properties, such as a greater AUC (Area Under the Curve).

For example, an expression construct encoding a fusion protein containing part or all of an antibody is mutagenized. Clones are generated, the corresponding proteins are expressed, and
20 the proteins are tested, either individually or in small pools, for relative sensitivity to proteases. Variant antibody fusion proteins with enhanced resistance to proteases are then tested for their pharmacokinetic properties, and a significant number of the protease-resistant antibody fusion protein variants have improved pharmacokinetic properties. The nucleic acids encoding the improved variant fusion proteins are sequenced, and some improved variants are found to
25 contain mutations at sites other than the fusion protein junction that cause the phenotype of enhanced resistance to proteolysis and improved pharmacokinetics.

Equivalents

30 The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are intended to be embraced therein.

Incorporation by Reference

Each of the patent documents and scientific publications disclosed herein is incorporated by reference into this application in its entirety.

What is claimed is:

- 1 1. An antibody-based fusion protein comprising an immunoglobulin (Ig) chain linked to a
2 non-Ig protein via a junction point, wherein said antibody-based fusion protein comprises
3 an amino acid alteration within 10 amino acids from said junction point, in said Ig chain
4 or said non-Ig protein, and wherein said antibody-based fusion protein has a longer
5 circulating half-life *in vivo* than a corresponding antibody-based fusion protein without
6 said amino acid alteration.
- 1 2. The fusion protein of claim 1 wherein the amino acid alteration increases the
2 hydrophobicity of said antibody-based fusion protein.
- 1 3. The fusion protein of claim 1 or 2 wherein said Ig chain is N-terminal to said non-Ig
2 protein.
- 1 4. The fusion protein of claim 1, 2 or 3 wherein said alteration changes the C-terminal
2 amino acid of the Ig chain.
- 1 5. The fusion protein of claim 1, wherein said non-Ig protein is a secreted protein.
- 1 6. The fusion protein of claim 5, wherein said non-Ig protein is a mature form of said
2 secreted protein.
- 1 7. The fusion protein of claim 1, wherein the Ig chain comprises part of an Ig heavy chain.
- 1 8. The antibody-based fusion protein of claim 7 wherein said Ig chain comprises at least the
2 CH2 domain of an IgG2 or an IgG4 constant region.
- 1 9. The antibody-based fusion protein of claim 7, wherein said Ig chain comprises at least a
2 portion of an IgG1 constant region having a mutation or a deletion at one or more amino
3 acids selected from the group consisting of Leu₂₃₄, Leu₂₃₅, Gly₂₃₆, Gly₂₃₇, Asn₂₉₇, and
4 Pro₃₃₁.
- 1 10. The antibody-based fusion protein of claim 7, wherein said Ig chain comprises at least a
2 portion of an IgG3 constant region having a mutation or a deletion at one or more amino
3 acids selected from the group consisting of Leu₂₈₁, Leu₂₈₂, Gly₂₈₃, Gly₂₈₄, Asn₃₄₄, and
4 Pro₃₇₈.
- 1 11. The antibody-based fusion protein of claim 7, wherein said Ig chain has binding affinity
2 for an immunoglobulin protection receptor.
- 1 12. The antibody-based fusion protein of claim 7, wherein said Ig chain has substantially
2 reduced binding affinity for a Fc receptor selected from the group consisting of FcγRI,
3 FcγRII and FcγRIII.

- 1 13. The antibody-based fusion protein of claim 7, wherein said non-Ig protein is selected
2 from the group consisting of a cytokine, a ligand-binding protein, and a protein toxin.
- 1 14. The antibody-based fusion protein of claim 13, wherein said cytokine is selected from the
2 group consisting of a tumor necrosis factor, an interleukin, and a lymphokine.
- 1 15. The antibody-based fusion protein of claim 14, wherein said tumor necrosis factor is
2 tumor necrosis factor alpha.
- 1 16. The antibody-based fusion protein of claim 14, wherein said interleukin is interleukin-2.
- 1 17. The antibody-based fusion protein of claim 14, wherein said lymphokine is a
2 lymphotoxin or a colony stimulating factor.
- 1 18. The antibody-based fusion protein of claim 11, wherein said colony stimulating factor is
2 a granulocyte-macrophage colony stimulating factor.
- 1 19. The antibody-based fusion protein of claim 13, wherein said ligand-binding protein is
2 selected from the group consisting of CD4, CTLA-4, TNF receptor, and an interleukin
3 receptor.
- 1 20. A method for increasing the circulating half-life of an antibody-based fusion protein
2 having an Ig chain linked to a non-Ig protein via a junction point, the method comprising
3 the step of substituting, deleting, inserting, or otherwise altering an amino acid at or near
4 said junction point.
- 1 21. The method of claim 20, wherein said fusion protein comprises a portion of a heavy
2 chain.
- 1 22. The method of claim 21, wherein said fusion protein comprises at least the CH2 domain
2 of an IgG2 or an IgG4 constant region.
- 1 23. The method of claim 20, 21, or 22, wherein said fusion protein comprises a heavy chain
2 moiety having a mutation that affects interaction with an Fc protection receptor.
- 1 24. The fusion protein of claim 1 comprising a linker between said Ig chain and said non-Ig
2 protein.
- 1 25. The fusion protein of claim 4, 5, 6, or 7, wherein said alteration is a substitution of one or
2 more amino acids.
- 1 26. An antibody-based fusion protein comprising
2 a) a first polypeptide comprising an Ig chain, and,
3 b) a second polypeptide comprising a non-Ig protein,
4 wherein said first polypeptide is joined to said second polypeptide to produce a junction
5 region having at least one mutation, and

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- 6 wherein said fusion protein has a longer circulating half life than a fusion protein having
7 a junction region without said mutation.
- 1 27. The fusion protein of claim 26, wherein said mutation is in the C-terminal portion of said
2 first polypeptide.
- 1 28. The fusion protein of claim 26, wherein said mutation is in the N-terminal portion of said
2 second polypeptide.
- 1 29. The fusion protein of claim 26 comprising a first mutation in the C-terminal portion of
2 said first polypeptide and a second mutation in the N-terminal portion of said second
3 polypeptide.
- 1 30. The fusion protein of claim 27 or 29 wherein said C-terminal portion comprises between
2 1 and 100 C-terminal amino acids of said first polypeptide.
- 1 31. The fusion protein of claim 30 wherein said C-terminal portion comprises between 1 and
2 10 C-terminal amino acids of said first polypeptide.
- 1 32. The fusion protein of claim 28 or 29 wherein said N-terminal portion comprises between
2 1 and 100 N-terminal amino acids of said second polypeptide.
- 1 33. The fusion protein of claim 32 wherein said N-terminal portion comprises between 1 and
2 10 N-terminal amino acids of said second polypeptide.
- 1 34. The fusion protein of claim 26 wherein said Ig is IgG1.
- 1 35. The fusion protein of claim 26 wherein said mutation is selected from the group
2 consisting of point mutations, deletions, insertions, and rearrangements.
- 1 36. The fusion protein of claim 34 wherein the C-terminal residue of said first polypeptide is
2 mutated to be an amino acid with a non-ionizable side chain.
- 1 37. The fusion protein of claim 36 wherein said C-terminal residue is a non-lysine amino
2 acid.
- 1 38. The fusion protein of claim 26 wherein said junction region consists of the C-terminal
2 region of said first polypeptide and the N-terminal region of said second polypeptide, and
3 wherein said mutation is present in one of said C-terminal and N-terminal regions.
- 1 39. The fusion protein of claim 26 wherein said junction region comprises a spacer or linker
2 peptide.
- 1 40. The fusion protein of claim 39 wherein said mutation consists of the presence of a spacer
2 or linker peptide between said first and said second polypeptides.
- 1 41. The fusion protein of claim 26 wherein said mutation is in a region that does not interact
2 with FcR or FcRp.

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- 1 42. A method for identifying a mutation that increases the circulating half life of an antibody-
2 based fusion protein having an Ig moiety and a non-Ig moiety comprising the steps of:
3 a) introducing a mutation in the region spanning the junction between the Ig
4 moiety and the non-Ig moiety;
5 b) comparing the serum half-lives of the antibody-based fusion protein with and
6 without a mutation; and,
7 c) selecting a mutation that increases the serum half-life of the antibody-based
8 fusion protein.
- 1 43. An antibody-based fusion protein comprising a mutation identified according to the
2 method of claim 42.
- 1 44. A method for treating a disease comprising the step of administering to a patient an
2 antibody-based fusion protein of claim 26.
- 1 45. The fusion protein of claim 1, 26, or 36, having a hydrophobic or non-polar amino acid
2 introduced via addition or substitution at or near said junction.
- 1 46. The fusion protein of claim 45, wherein said amino acid is selected from the group
2 consisting of Leu, Ala, Trp, and Gly.
- 1 47. The fusion protein of claim 48, wherein said amino acid is Ala.

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Pharmacokinetics of KS-IL-2 with junction
alterations (IL-2 ELISA)

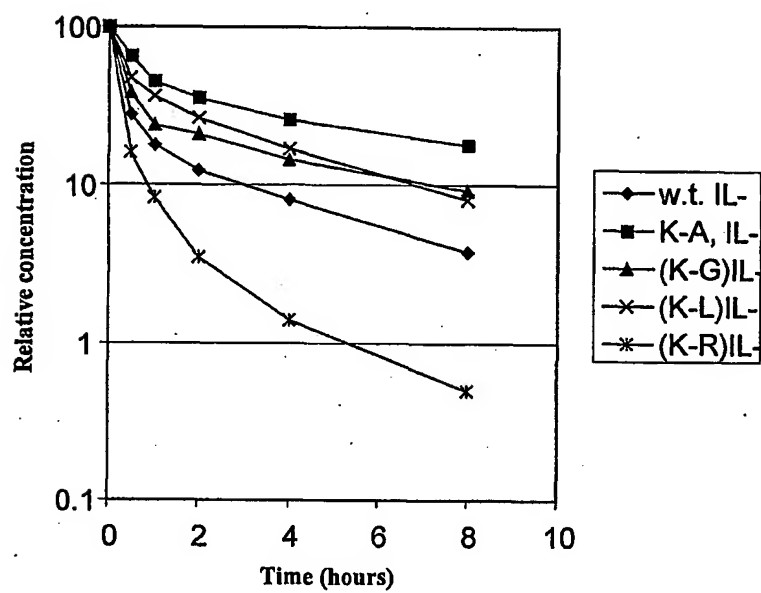


Figure 1A

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**Effect of alteration of fusion junction on
pharmacokinetics of KS-IL-2 (Fc ELISA)**

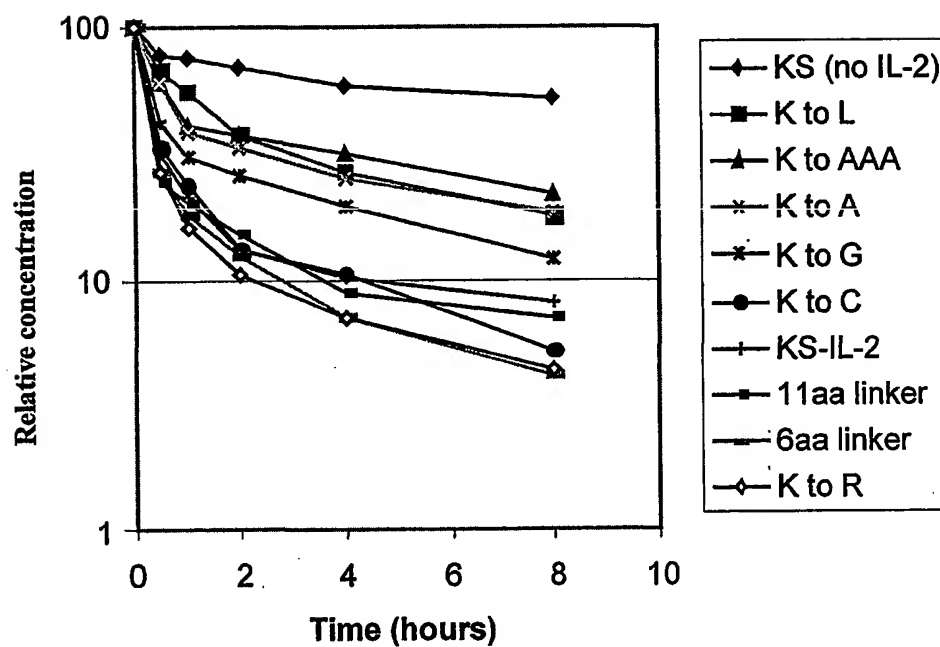
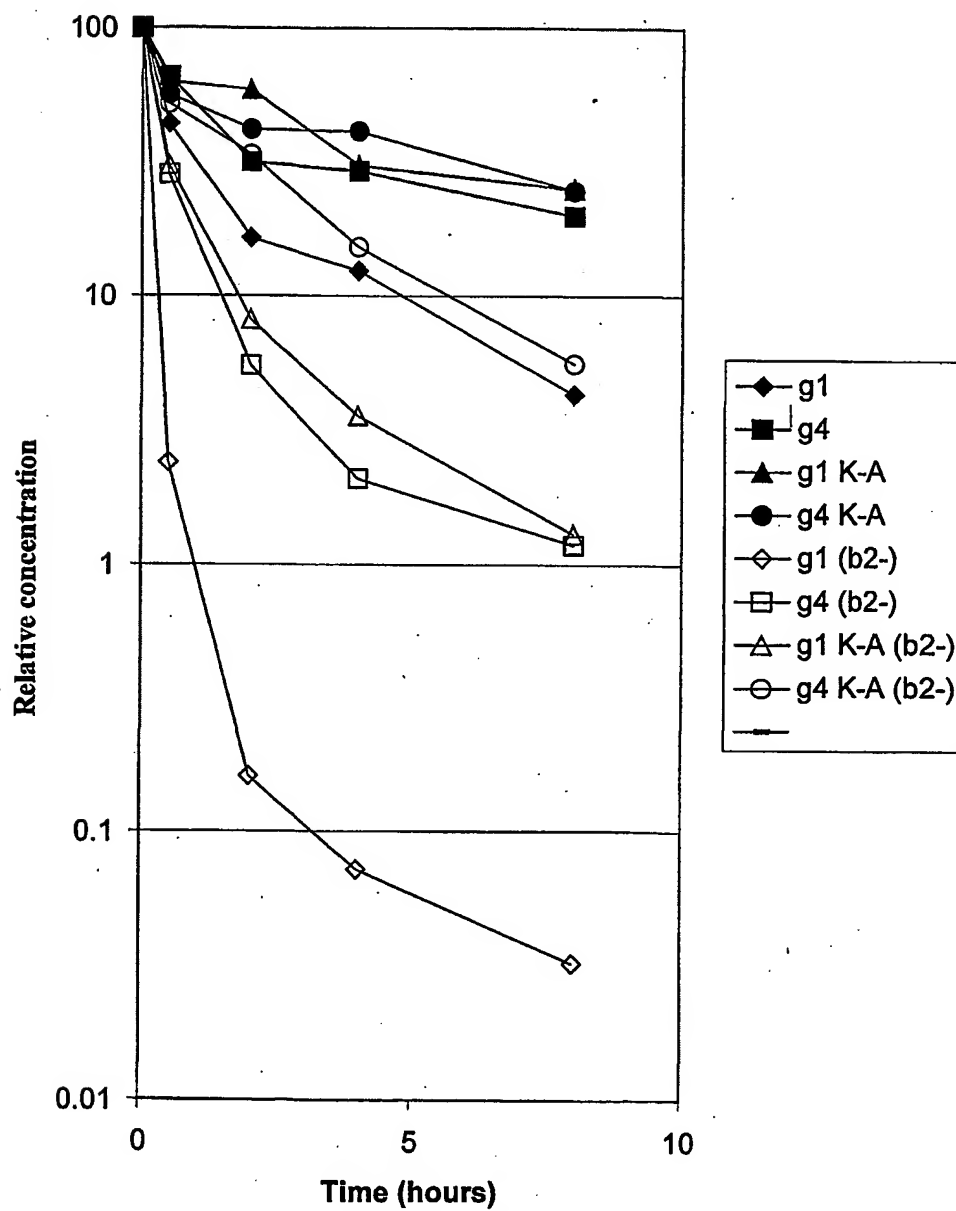


Figure 1B

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Pharmacokinetics of KS-IL-2 fusions in wild-type and beta2 mutant mice

**Figure 2**

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Pharmacokinetics of antibody-TNFalpha

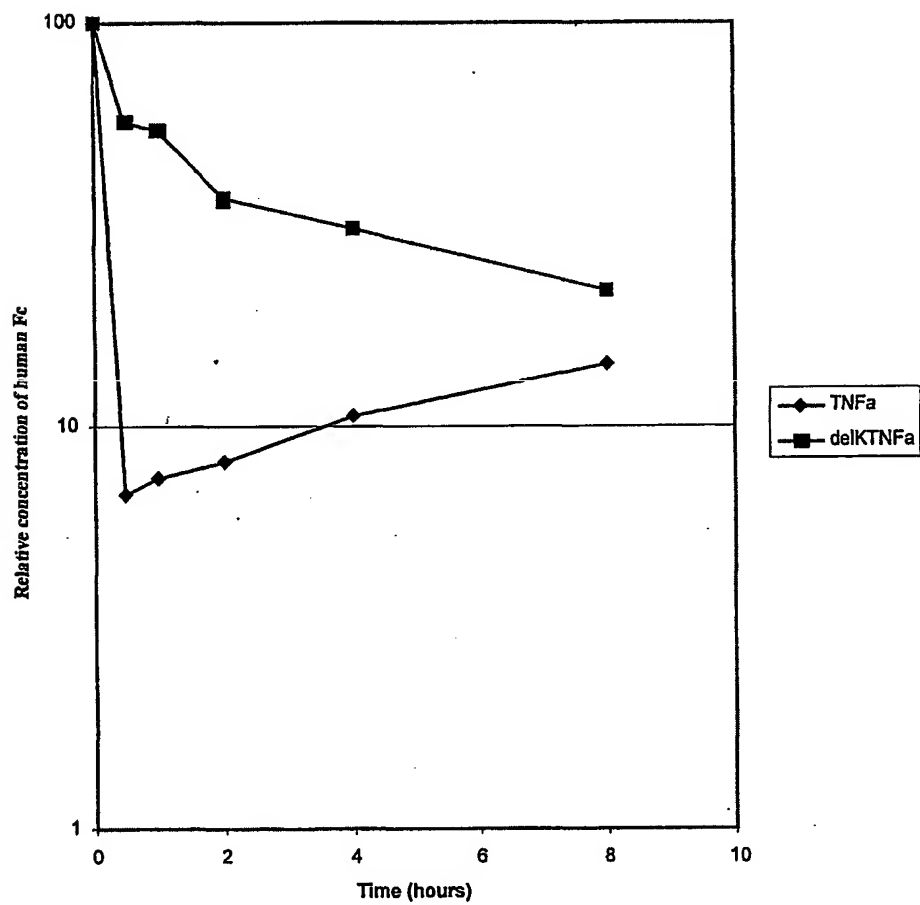


Figure 3

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Binding of antibody-IL-2 fusion proteins to Fc receptor

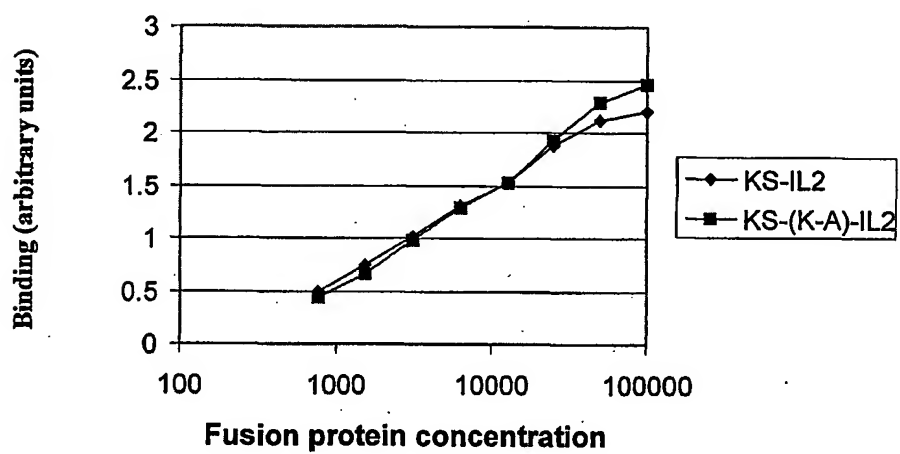
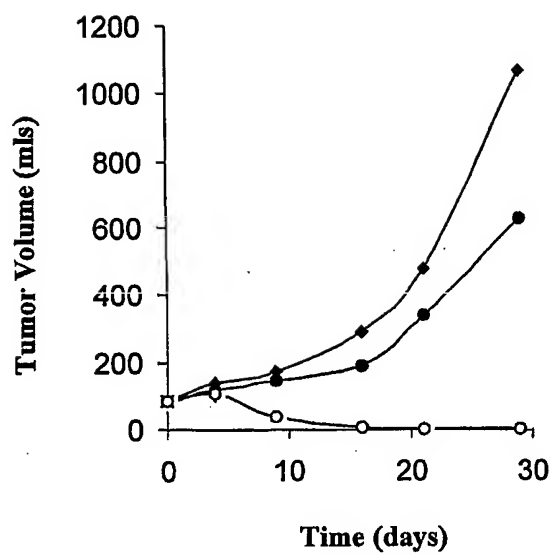


Figure 4 (J774 binding)

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**Figure 5**

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- (74) Agent: WALLER, Patrick, R., H.: Testa, Hurwitz & Thibault, L.L.P., High Street Tower, 125 High Street, Boston, MA 02110 (US).
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(54) Title: ENHANCING THE CIRCULATING HALF-LIFE OF ANTIBODY-BASED FUSION PROTEINS

(57) Abstract: Disclosed are compositions and methods for enhancing the circulating half-life of antibody-based fusion proteins. Disclosed methods and compositions rely on altering the amino acid sequence of the junction region between the antibody moiety and the fused protein moiety in an antibody-based fusion protein. An antibody-based fusion protein with an altered amino acid sequence in the junction region has a greater circulating half-life when administered to a mammal. Disclosed methods and compositions are particularly useful for reducing tumor size and metastasis in a mammal.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/04455

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K19/00 G01N33/68 //C07K16/00.C07K16/28.C07K14/52.
C07K14/525.C07K14/55

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EMBASE, CHEM ABS Data, WPI Data, PAJ, EPO-Internal, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

3 January 2002

Date of mailing of the international search report

18/01/2002

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Fax: (+31-70) 340-3016

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INTERNATIONAL SEARCH REPORT

In. tional Application No
PCT/US 01/04455

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No |
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In International Application No

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| | | BR 9908226 A | 24-10-2000 |
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| | | HU 0100813 A2 | 28-06-2001 |
| | | NO 20004218 A | 24-10-2000 |
| | | PL 342497 A1 | 04-06-2001 |
| | | WO 9943713 A1 | 02-09-1999 |